CERTIFICATE OF MAILING
37 C.F.R. §1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date below:

February 13, 2002
Date

Steven L. Highlander

#18 Decl. w/ attach. 3/12/02 ecl

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shuyuan ZHANG *et al.*

Serial No.: 09/203,078

Filed: December 1, 1998

For: METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

Group Art Unit: 1648

Examiner: S. Foley

Atty. Dkt. No.: INRP:081

DECLARATION OF SHAWN GALLAGHER UNDER 37 C.F.R. §1.132

Hon. Commissioner for Patents
Washington, D.C. 20231

I, Shawn Gallagher, do declare that:

1. I am a citizen of the United States residing at 1730 Shoreline Drive, Missouri City, TX, 77459. I am an employee of Introgen Therapeutics, Inc. ("Introgen"), and a named inventor for the above-captioned application.

2. I have been employed by Introgen for 5 years and currently hold the position of Vice President of Product Development. My duties at Introgen include overseeing and management

of development of our adenoviral gene therapy products, one of which, adenoviral p53 ("Advexin®") is currently in phase III registration trials. I am also in charge of managing our clinical manufacturing facility, which is currently committed to the preparation of pharmaceutical grade adenoviral gene therapy products. I have extensive experience in adenovirus purification and pharmaceutical preparation, as evidenced by my attached *curriuculum vitae* [Exhibit 1].

3. In providing this declaration, I have reviewed the Office Action mailed on September 13, 2001. I am also familiar with the content of the above-captioned application, as well as with the pending claims.

4. Based on my reading of the Office Action, it appears that the examiner has misunderstood the present claims. The Action makes several references to the "cell cycle" in discussing the pending claims. However, there is no mention of the cell cycle in the pending claims but, rather, a recitation of "mid-log" and "stationary growth" phases. These terms refer to phases of ***a cell population growth curve***, and not to phases of the cell cycle, which are M (mitotic), G_1 (gap), S (synthesis) and G_2 (post-S, pre-M).[1] In this regard, the specification at pages 20-22, is instructive:

> In any cell culture system, there is a characteristic growth pattern following inoculation that includes a lag phase, an accelerated growth phase, an exponential or "log" phase, a negative growth acceleration phase and a plateau or stationary phase. The log and plateau phases give vital information about the cell line, the population doubling time during log growth, the growth rate, and the maximum cell density achieved in plateau. ***In the log phase, as growth continues, the cells reach their maximum rate of cell division.*** Numbers of cells increase in log relationship to time. During this period of

[1] Molecular Biology of the Cell, Alberts *et al.*, eds., Garland Publishing, NY, 1983, pp. 611-612 (Exhibit 2).

that the skilled artisan can formulate growth curves for any such cell line and identify the aforementioned regions on the curve.

Specification at page 20, line 22, to page 22, line 27 (emphasis added). As illustrated by the highlighted portions, the terms "mid-log" and "stationary" phase have nothing to do with the cell cycle.

5. In light of the foregoing explanation of the terms "mid-log" and "stationary growth" phases, and based on factual information about the growth of cells in culture, it is my conclusion that the Huyghe *et al.* (1995; C44) paper, for the reasons discussed below, does not disclose our invention.

6. In the Huyghe *et al.* reference at page 1404, in the second paragraph under "MATERIALS AND METHODS," the article indicates that the cell monolayers were at "50-60% confluency" when infected with adenovirus. However, there is no information provided on the seeding density, lag phase or the doubling times for 293 cells under the authors' care, such that would permit a determination of whether these cells were in "mid-log" to "stationary growth" phases at the time the were infected – 2 to 2.5 days after seeding. Without knowledge of seeding density, lag phase or doubling times for the cells used in that study, there is no way one can conclude that Huyghe *et al.* infected the cells between mid-log and stationary phase.

7. At best, one can merely estimate the "phase" of the culture described in the Huyghe *et al.* reference, making certain assumptions and extrapolations, when infected at 2-2.5 days and 50-60% confluency. If one assumes that Huyghe *et al.* seeded at a seeding density of approximately

1-3 x 10^4 cells/cm^2, and that the 293 cells employed by Huyghe *et al.* had a lag phase of approximately 24 hours and a doubling time of approximately 36 hours, then one can calculate the phase at 50-60% confluency as early log phase, certainly less than mid-log phase, using the following calculations:

- **Initial density (midpoint of assumption range) = 2 x 10^4 cells/cm^2**
- **Growth period (2.5 days – lag time) = 1.5 days = 36 hours**
- **With a doubling time of 36 hours, the cells population will double once, giving final concentration equal to 2 x 10^4 cells/cm^2 x 2 = 4 x 10^4 cells/cm^2, consistent with early log phase density**

8. There are a number of scientific publications that support the foregoing conclusion. First, I would direct the examiner to Freshney, R.I., "Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed." [Exhibit 3]. At page 240, it is stated that "[t]oward the end of the log phase, the culture becomes confluent—*i.e.*, all the available growth surface is occupied and all the cells are in contact with surrounding cells." Based on this statement, it seems unlikely that Huyghe *et al.*'s cells, at 50-60% confluency, would be in late log phase.

9. In MediaTech's Technical Information bulletin [Exhibit 4], it is indicated, in the first paragraph of column 2 on the first page, that in order to ensure that cultures are in log phase, they must be at least 70% confluent. Thus, a culture that is only 50-60% confluent like that of Huyghe *et al.* is likely not in log phase, or at the very least, only in early log phase, not mid-log or late-log phase.

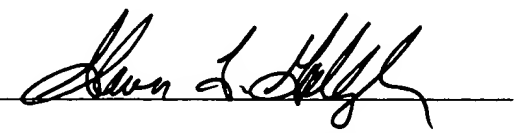
10. Next, I refer to the article of Kuchler, "Biochemical Methods in Cell Culture and Virology" [Exhibit 5]. At page 90, the lag phase, which precedes the log phase, is said to vary from 24 to 48 hours. Given that Huyghe *et al.* infected cells between 48-60 hours after seeding, Kuchler suggests that Huyghe *et al.*'s cells would be barely out of lag phase. While specific cell lines or strains of cell lines can demonstrate significant variation in characteristic doubling times and duration of lag phase, our own experience with 293 cells from various sources indicates that lag times of 24-48 hours are not uncommon after passaging.

11. Although none of the preceding evidence presents conclusive proof as to the precise point in the population growth curve at which Huyghe *et al.* infected cells, it is my opinion, based on the available evidence and my sample calculation, that the cells infected by Huyghe *et al.* were likely, at the very latest, in early log phase, and not within the "mid-log phase to stationary growth phase" as specified by the pending claims.

12. I hereby declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the referenced patent application or any patent issued thereon.

12 Feb 2002

Date



Shawn Gallagher, M.S.

SHAWN L. GALLAGHER
1730 Shoreline Drive
Missouri City, TX 77459
(281) 499-8588; e-mail: sggolfnut@aol.com

CAREER SUMMARY:

High energy, team player with an extensive history of successful program management in the following areas:

- cGMP manufacturing
- Facility design and construction
- Process development (fermentation and purification)
- Contract negotiation and management
- Strategic planning
- Validation

EXPERIENCE:

1996 to Present **INTROGEN THERAPEUTICS, INC.** **Houston, TX**
A leading biopharmaceutical company in the commercialization of gene based therapeutics.

Vice President, Product Development **1999-present**
Vice President, Manufacturing **1996-1999**

- Corporate Officer responsible for manufacturing and quality systems (QA/QC) for the cGMP production of viral vectors in support of cancer gene therapy clinical program.
- Directed the creation of a cGMP infrastructure to facilitate the transition of novel technology from an academic setting (at M.D. Anderson Cancer Center) to commercial development.
- Project Executive for the design of a new commercial manufacturing facility currently under construction.
- Lead the process engineering team in the development and implementation of a scalable manufacturing process for adenoviral based viral vectors.
- Primary technical liaison with pharmaceutical partner (RPR).

1995 to 1996 **MAGENTA CORPORATION** **Rockville, MD**
The foremost contract manufacturing organization servicing the gene therapy industry with the production of viral vectors for clinical use.

Director of Operations

- Coordinated the activities for two functional groups providing the gene therapy and broader biopharmaceutical industries with contract manufacturing services. These two groups, Virus Production and Cell Banking, occupy four dedicated cGMP manufacturing suites and apply

large scale mammalian and insect cell culture techniques to varied manufacturing problems.

- Interfaced with clients from initial contact phase through technology transfer until completion of production and testing.

support for production.

1985 to 1987 **UNIVERSITY OF CALIFORNIA AT SAN DIEGO** **La Jolla, CA**

Research Assistant /Teaching Assistant, Departments of Chemistry and AMES

EDUCATION:

MS, Chemical Engineering, University of California at San Diego, 1987.

- Investigation of the physiological behavior of mammalian cells.
- Design, testing, and modeling of novel hollow-fiber bioreactor systems.

BS, Chemical Engineering, University of Colorado, Boulder, 1985.

- Study of the growth kinetics of *Saccharomyces cerevisiae* in chemically defined media.

AWARD: 1989 Kirkpatrick Chemical Engineering Achievement Award.

BioTechnetics was an honor award recipient. This award is given every two years in recognition of outstanding chemical engineering technology, successfully commercialized and achieved through group effort. The biochemical engineering group was honored for the development of novel bioreactor systems. As a key member of the BioTechnetics process development team, I played an integral role in the successful commercialization of the bioreactor technology.

Chemical Engineering 96,12:79, 1989.

PROFESSIONAL AFFILIATIONS:

AIChE, ACS, ESACT, ISPE

REFERENCES:

Available on request

MOLECULAR BIOLOGY OF THE CELL

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson

Garland Publishing, Inc.
New York & London

"Long ago it became evident that the key to every biological problem must finally be sought in the cell, for every living organism is, or at sometime has been, a cell."

Edmund B. Wilson
The Cell in Development and Heredity
3rd edition, 1925, Macmillan, Inc.

Bruce Alberts received his Ph.D. from Harvard University and is currently a Professor in the Department of Biophysics and Biochemistry at the University of California Medical School in San Francisco. *Dennis Bray* received his Ph.D. from the Massachusetts Institute of Technology and is currently a Senior Scientist in the Medical Research Council Cell Biophysics Unit at King's College London. *Julian Lewis* received his D.Phil. from Oxford University and is currently a Lecturer in the Anatomy Department at King's College London. *Martin Raff* received his M.D. degree from McGill University and is currently a Professor in the Zoology Department at University College London. *Keith Roberts* received his Ph.D. from Cambridge University and is currently Head of the Department of Cell Biology at the John Innes Institute, Norwich. *James D. Watson* received his Ph.D. from the University of Indiana and is currently Director of the Cold Spring Harbor Laboratory. He is the author of *Molecular Biology of the Gene* and, with Francis Crick and Maurice Wilkins, won the Nobel Prize in Medicine and Physiology in 1962.

Cover photograph kindly provided by Michael Verderame and Robert Pollack of Columbia University. The fluorescein-phalloidin used to stain the actin cables was the generous gift of Drs. Theodor Wieland and A. Deboben of the Max Planck Institute, West Germany. The photograph is of a mouse fibroblast that had been transformed to anchorage-independent growth by the virus Simian Virus 40 (SV40) and subsequently selected for anchorage-dependent growth. This particular cell was stained for SV40 large T antigen (*red*) and fluorescein-phalloidin (*green*), which specifically stains F actin.

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Cell Growth and Division

11

As highly organized units in a universe favoring disorder, cells are subject to wear and tear as well as to accidents. Any individual cell is therefore bound to die. If an organism is to continue to live, it must create new cells at a rate as fast as that at which its cells die. For this reason, cell division is central to the life of all organisms. In an adult human, for example, millions of cells must divide every second simply to maintain the status quo.

The process of cell division itself is strikingly visible in the microscope; it consists of two sequential processes: nuclear division (called **mitosis**) and cytoplasmic division (called **cytokinesis**). But before a typical cell can divide, it must double its mass and duplicate all of its contents. Only in this way will the two new daughter cells contain all of the components that they need to begin their own cycle of cell growth followed by division. Most of the work involved in preparing for division goes on invisibly during the growth phase of the cell cycle, which is, quite misleadingly, denoted as **interphase.**

Although a cell spends most of its lifetime in interphase and only occasional periods in the cell-division phase, most early work on the cell cycle focused on the brief division events (mitosis and cytokinesis), largely because they could be studied by direct microscopic examination. More recently, through the use of more indirect and sophisticated techniques, we have learned a considerable amount about the interphase part of the cell cycle as well. In this chapter we shall describe some of the methods currently used to study the cell cycle, consider cell-cycle regulation, and discuss several of the main events occurring during each of its different phases. Although our knowledge of the molecular basis of the cell cycle is fragmentary, wherever possible we shall try to discuss the mechanisms that are likely to be involved.

The Control of Cell Division[1,2]

Most cell components are made continuously throughout the interphase period between cell divisions. It is, therefore, difficult to define distinct stages in the progression of the growing cell through interphase. One outstanding exception is DNA synthesis, since the DNA in the cell nucleus is replicated only

Second Edition

CULTURE OF ANIMAL CELLS

A Manual of Basic Technique

R. Ian Freshney

Department of Medical Oncology
Cancer Research Campaign Laboratories
University of Glasgow

Alan R. Liss, Inc., New York

count the cells in three wells of each plate: (a) remove medium completely from wells to be counted; (b) add 1 ml trypsin to each well; (3) incubate with trypsin; and (4) after 15 min, disperse cells in trypsin and transfer 0.4 ml to counting fluid and count on cell counter.
Note. Hemocytometer counting may be used but may be difficult at lower cell concentrations. Reduce trypsin volume to 0.1 ml and disperse cells carefully without frothing using a micropipette and transfer to hemocytometer.
6.
Return plate to incubator as soon as cell samples in trypsin are removed. The plate must be out of the incubator for the minimum length of time, to avoid disruption of normal growth.
7.
Repeat sampling at 48 and 72 hr as in steps 5 and 6.
8.
Change medium at 72 hr or sooner if indicated by pH drop (see above).
9.
Continue sampling at daily intervals for rapidly growing cells (doubling time 12–14 hr) but reduce frequency of sampling to every 2 days for slowly growing cells (doubling time > 24 hr), until plateau is reached.
10.
Keep changing medium every 1, 2, or 3 days as indicated by pH.

Analysis

1. Calculate cell number per well, per ml of culture medium (same figure), and per cm^2 of available growth surface in well. (Stain one or two wells (see Chapter 13) at each density to determine whether distribution of cells in wells is uniform and whether they grow up the sides of the well.)

2. Plot cell density (per cm^2) and cell concentration (per ml), on a log scale, against time on a linear scale (Fig. 10.3).

3. Determine the lag time, population doubling time, and plateau density (see below and Fig 10.3)

4. Establish which is the appropriate starting density for routine passage. Repeat growth curve at intermediate cell concentrations if necessary

Variations

1. Different culture vessels may be used, e.g., 25 cm^2 flasks, although more cells and medium will be required, or flat-bottomed glass-sample tubes. Individual tubes have the advantage that the rest are not disturbed when samples are removed for counting.

2. Frequency of medium changing may be altered.

3. Different media or supplements may be tested.

Suspension cultures

1.
Add cell suspension in growth medium to wells at a range of concentrations as for monolayer.
2.
Sample 0.4 ml at intervals as per trypsin samples. Alternatively, seed two 75-cm^2 flasks with 20 ml for each cell concentration and sample 0.4 ml from each flask daily or as required. Mix well before sampling and keep flasks out of incubator for the minimum length of time. Do not feed cultures during growth curve.

The growth cycle (Fig. 10.3) is conventionally divided into three phases.

The Lag Phase

This is the time following subculture and reseeding during which there is little evidence of an increase in cell number. It is a period of adaptation during which the cell replaces elements of the glycocalyx lost during trypsinization, attaches to the substrate, and spreads out. During spreading the cytoskeleton reappears and its reappearance is probably an integral part of the spreading process. Enzymes, such as DNA polymerase, increase, followed by the synthesis of new DNA and structural proteins. Some specialized cell products may disappear and not reappear until cessation of cell proliferation at high cell density.

The Log Phase

This is the period of exponential increase in cell number following the lag period and terminating one or two doublings after confluence is reached. The length of the log phase depends on the seeding density, the growth rate of the cells, and the density at which cell proliferation is inhibited by density. In the log phase the growth fraction is high (usually 90–100%) and the culture is in its most reproducible form. It is the optimal time for sampling since the population is at its most uniform and viability is high. The cells are, however, randomly distributed in the cell cycle and, for some purposes, may need to be synchronized (see Chapter 23).

Guide to Subculturing Cell Cultures

Morphology Each cell line or cell type expresses different characteristics in terms of growth and appearance in culture. Many cell lines grow as a single sheet monolayer attached to both themselves, and the culture vessel. Other cell types exist as single cells or clumps of cells suspended in the growth medium. Both adherent and suspension cultures must be maintained regularly to prevent overgrowth and accelerated cell death from exhausted medium and to promote the growth of the next generation of cells.

Cell Dissociation Viable subcultures may be obtained by transferring a particular volume of cells to new culture vessels with fresh medium. These fresh cultures are allowed to grow and divide as normal until such time the culture reaches confluence and the cells are used for experiments or subcultured. To do this correctly, obtain a single-cell suspension first. For adherent cell types, proteolytic enzymes, such as trypsin (MT Catalog Numbers 25-050, 25-052, 25-053, and 25-054), are used to break cell-cell and cell-substrate bonds and create a suspension from which new cultures may be split. For cultures already growing in suspension, this enzyme step is not necessary.

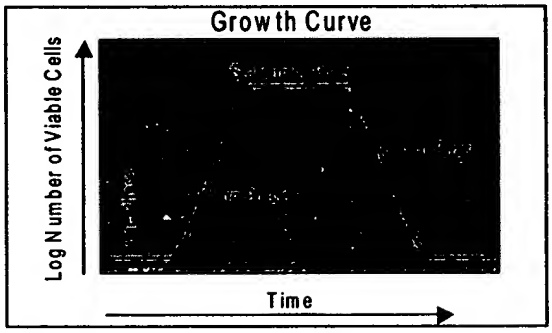
Harvesting describes the detachment of adherent cell lines to prepare a cell suspension for counting. During this step, the intercellular and intracellular (cell-substrate) bonds are broken, allowing the cells to separate into a single cell suspension. Depending on the cell type and the culture environment, this is achieved by using enzymatic or non-enzymatic dissociation solutions, such as Cellstripperâ , catalog number 25-056.

Growth Phases Cell growth typically exhibits a consistent pattern comprising three main phases, including an initial lag phase, a period of logarithmic growth, and a final stationary phase. See Figure 1. This growth pattern continues for each subculture despite the cell type. The initial lag phase occurs at the beginning of a subculture as the cells become accustomed to the new environment, during which they do not divide. The length of the log phase is determined by cell conditions prior to subculturing, as well as the seeding density and changes in the growth medium. The log (logarithmic) phase is a period of active proliferation, during which the number of cells increases exponentially. The length of the log phase is determined by many factors, including the seeding density and rate of normal cell growth, as well factors affecting the lag phase. The final stationary phase occurs when the rate of cell proliferation slows down. During this phase, the rate of cell division may be balanced by the rate of cell death, thus showing no change in cell density.

Subculturing is usually performed during the log phase when the cells are at their healthiest and are able to adapt to the new environment most efficiently. This is also the best time for cryopreservation and functionality studies. Check for cultures that appear at least 70% confluent.

Culture Examination Before handling, it is good practice to observe cultures both microscopically and macroscopically. Visual observations of the culture flask and medium may indicate evidence of microbial contamination including pH fluctuations and turbidity, as well as fungal colonies. The monolayer may also be viewed macroscopically to obtain a general idea of confluence. See Figures 2 and 3 below. This is most easily performed by viewing the culture vessel against a light source. Further microscopic observation may substantiate abnormal cell appearance and confirm microbial contamination. Rounding cells may indicate mitosis, especially if the cells are very refractive, or bright. Dead cells do not express this same brightness.

Figure 1. Growth Phases of Cells in Culture



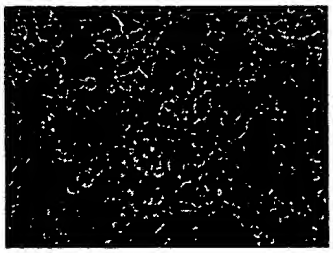


Figure 2. Monolayer
Confluent Monolayer. This culture is ready for splitting.

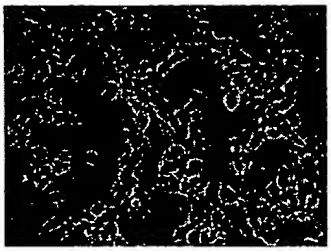


Figure 3. Monolayer
This monolayer appears less confluent than the monolayer in Figure 1. A cell count would determine if maximum cell density has been reached.

Routine Subcultivation & Maintenance

Procedure The following procedure describes the basic principles involved in the routine subcultivation and maintenance of cell cultures. It **is imperative that aseptic technique is maintained throughout this procedure.**

1. Visually examine cultures, as discussed above.

2. Harvest adherent cell lines using a dissociating agent. For suspension lines, this step is not required. Proceed to Step 7.

3. Aspirate and discard the culture medium.

4. Rinse the monolayer with the dissociation solution or a buffered salt solution without calcium and magnesium. This rinsing step removes any residual serum from the monolayer that could inactivate the trypsin. Use about 3ml for 25-cm^2 flasks and 5ml for 75-cm^2 flasks. Be sure to add the solution on the side of the flask opposite the monolayer to prevent cell loss. Swirl the solution gently across the cell sheet. Remove and discard the solution.

5. Repeat the washing step, this time using the dissociation solution. For more fastidious cell lines, the flask can be placed in a 37°C incubator to facilitate the enzyme. The flask may also be observed microscopically to monitor progress and prevent over-exposure of the cells to the enzyme activity. Non-enzymatic solutions, such as Cellstripperâ , will require longer incubation times than when using enzymes like trypsin, but are more gentle to the cells.

6. Once the cells are detached, add the desired amount of growth medium to the flask, creating a cell suspension. When pipetting the growth medium into the flask, be sure to "wash" the sides of the flask to ensure all cells become suspended. It is not unusual for a small amount of cells to remain attached to the flask or substrate. However, more vigorous pipetting may be necessary to break up cell clumps or to aid in the removal of attached cells.

7. Using the cell suspension, determine the appropriate inoculum for subculturing the particular cell line. This seeding density may be determined by performing growth rate studies, or by counting. When simply passing a culture, when tracking exact cell densities in not necessary, a split according to a suspension ratio is commonly used. For example, a 1:2 ratio indicates that the cell suspension may be split in half between two culture flasks of equal surface area. Counting, however, requires a hemacytometer or other cell counting device. A hemacytometer is used in conjunction with a stain, such as trypan blue (MT 25-900-CI). Simply remove a small amount of the cell suspension, such as 500ml, and mix with an equal amount of trypanblue. Using a clean hemacytometer, determine the number of cells/ml in the suspension and calculate the volume of suspension required to seed the desired density for each subculture.

8. Dispense these aliquots into clean, sterile, labeled culture vessels. Add the desired amount of culture medium to the vessels and pipette to ensure equal distribution of cells.

9. Return cultures to their appropriate environment. Most mammalian cell lines require a 37°C growth environment including a carbon dioxide level of 5%. The type of culture medium may alter the type of environment required for cell growth.

10. After about 24hours, observe the culture for reattachment and active growth. Note any unusual observations. Change medium as needed and subculture when necessary.

References:

1. Davis, J.M. *Basic Cell Culture.* Oxford University Press, New York, 93-134 (1996).
2. Doyle, A. and J. Bryan Griffiths. *Mammalian Cell Cuture.* John Wiley & Sons, West Sussex, UK, 47-48 (1997).
3. Freshney, R.I. *Animal Cell Culture: A Practical Approach.* Oxford University Press, New York, 62-64 (1992).
4. Freshney, R.I. *Culture of Animal Cells: A Manual of Basic Technique, 4th Ed.* Wiley Liss, New York, (2000).

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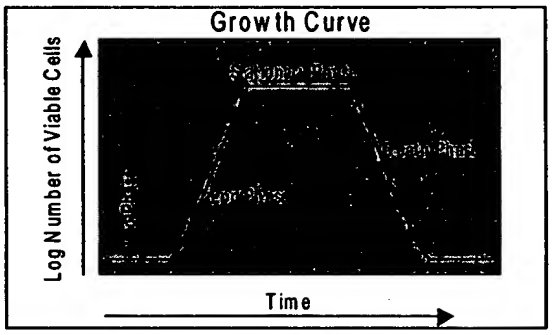

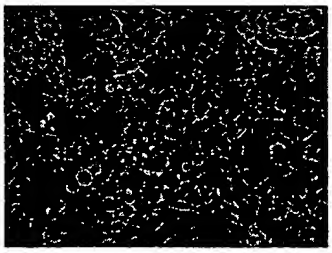

Figure 2. Monolayer
Confluent Monolayer. This culture is ready for splitting.

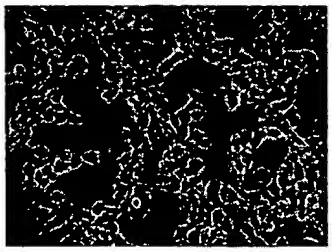

Figure 3. Monolayer
This monolayer appears less confluent than the monolayer in Figure 1. A cell count would determine if maximum cell density has been reached.